Model systems and the elements of behavior

–Neural mechanisms of learning and memory

[In which we move from genetics to neurobiology and see commonalities in the way snails, flies, and mice remember]

Heritability testing, gene–environment correlations and interactions, genetic mapping by linkage and association, then genome sequences, catalogues of every gene, every genetic variant, genomic technology that consumes a budget large enough to run a small country, the weight of 21st-century molecular genetics is enough to win an argument that genes produce behavior. But of course they don't. Genes, in fact, merely serve as a library that each cell draws upon when it needs to make proteins. Behavior comes out of the ensemble of cellular activities, and the cells that usually matter most are those in the nervous system. Genetic variations that affect behavior often do so by modifying the activity of neurons, and such modifications result from altering the timing, placement, amount, or effectiveness of a gene's product. The relevant neurons, in turn, take part in the brain's complex circuitry. So we need to look at circuits to understand how behavior is produced out of genetic variation.

This is easier said than done, especially for brains as complex as those in humans, mice, and even fruit flies, with 100 billion, 100 million, and 100,000 neurons, respectively. Circuit 'breaking,' as it is affectionately known in the business, is more easily accomplished in marine crustaceans and molluscs, which tend to have relatively simple nervous systems with large, recognizable neurons. These creatures are not what we would call geniuses, with the possible exception of the octopus, but they do behave. They have to, in order to get by in this world. Some of these behaviors are pretty simple, but then if we can't understand simple behaviors, how can we hope to explain complex ones?

Marine invertebrates: using a sea snail to learn about memory

Why anyone would expect a sea snail to teach us about learning and memory is pretty

mysterious. It's not as if this invertebrate is famous for its powers of remembrance. People don't quote its infallible powers of recall. In fact, most people wouldn't recognize one. Furthermore, even assuming a giant marine invertebrate can remember where you left your car keys, is it really likely that something so simple is going tell us anything about something as complex as the mammalian brain? Our own cognitive abilities so dwarf those of invertebrates it looks a sure bet we have a type of neuronal organization qualitatively different from that found in invertebrates. Who on earth ever thought this animal would be a useful model organism?

Eric Kandel did. Eric Kandel, born in Austria before Hitler annexed it, still remembers Kristallnacht as if it were yesterday:

> **Of all the cities under Nazi control, the destructiveness in Vienna on Kristallnacht was particularly wanton. Jews were taunted and brutally beaten, expelled from their businesses, and temporarily evicted from their homes so that both could be looted by their neighbors. My father was rounded up by the police together with hundreds of other Jewish men... My early experiences in Vienna almost certainly contributed to my curiosity about the contradictions and complexities of human behavior. How are we to understand the sudden release of such great viciousness in so many people? How could a highly educated and cultured society, a society that at one historical moment nourished the music of Haydn, Mozart, and Beethoven, in the next historical moment sink into barbarism?**

The reason Kandel chose the sea snail (*Aplysia*) is the same reason that drove Benzer to use mutagenesis in the fly: a chance to reduce the problem of behavior to its bare essentials. Kandel took note of the breakthroughs in understanding how nerve cells work by studying squid giant axons, the nerve–muscle synapse of the frog, and the eye of the horseshoe crab, *Limulus*.

Despite discouragement from many of his senior colleagues, Kandel persisted in trying to find an invertebrate that would help:

> **I believed that any insight into the modification of behavior by experience, no matter how simple the animal or the task, would prove to be highly informative. After an extensive search that included crayfish, lobster, flies, and the nematode worm Ascaris, I settled on Aplysia, the giant marine snail. Aplysia offered three major technical advantages: (1) its nervous system has a small number of cells, (2) the cells are unusually large, and, as I realized with time, (3) many of the cells are invariant and identifiable as unique individuals.**

Note that having a good memory doesn't figure in the list, and note also that *Aplysia* is not a genetic model organism. Kandel was not considering a genetic assault on the problem. That was to come later.

Key Paper

Kandel, E.R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science* **294**:1030–1038.

An introduction to the neurobiology of *Aplysia* and similar discoveries made in other organisms.

Aplysia does learn things, although admittedly not a lot. When a wave comes crashing in, it learns to close its gill opening. The neuronal circuitry is simple—a set of sensory neurons capable of detecting water on the gill connects to a set of motor neurons capable of driving muscles that withdraw the gill (Figure 10.1). Gill shutting is a defensive reaction that occurs

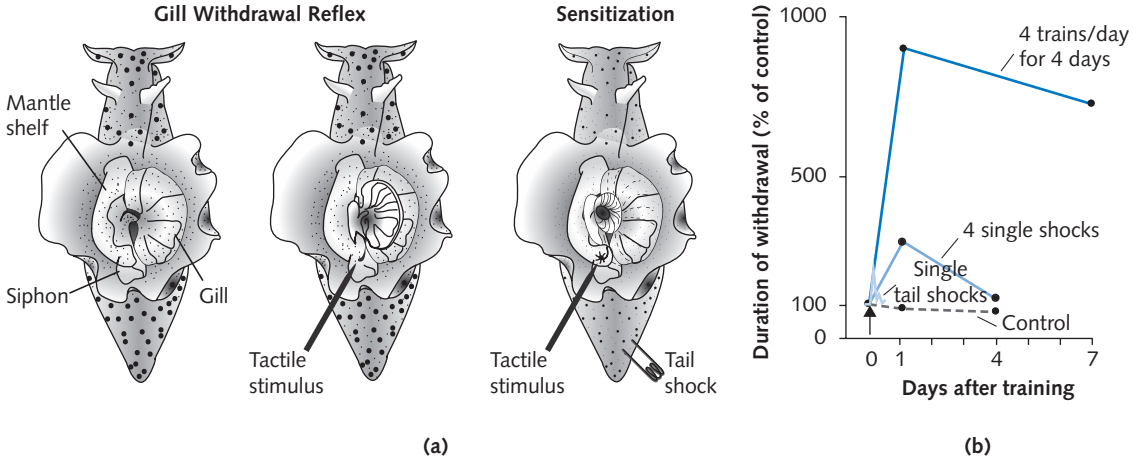

Figure 10.1 A dorsal view of *Aplysia* showing the gill, the animal's respiratory organ.
Source: Kandel (2001).

in response to any mechanical stimulus. If the gill is repeatedly stimulated, *Aplysia* becomes more blasé and gradually responds less and less. On the other hand, if something alarming happens, such as a bite or a blow to some other part of its body or an electric shock, the snail becomes hypersensitive to any kind of gill stimulation. A single shock gives rise to a memory lasting only a few minutes, but four or five spaced shocks gives rise to a memory that lasts several days (Figure 10.1). Thus, as in *Drosophila*, and in our own species, there is both short- and long-term memory.

Kandel and his colleagues were able to define the neural circuitry involved in this response. *Aplysia* has about 20,000 neurons, a fifth the number found in the fly. The cells are organized into ten anatomical units called ganglia, each containing about 2,000 cells. The gill reflex has 24 mechanoreceptor sensory neurons that pass information from the skin directly to six motor cells that cause the gill to withdraw. In addition, there is an indirect path from this sensory input to motor output via interneurons. The pathway turned out to be invariant: in each animal, the same neurons made the same connections. This turned out to be true for other behaviors as well (e.g. locomotion, feeding, and the other exciting activities of sea snails). Precise wiring raises a problem for learning—how can a flexible behavior be hard-wired?

The answer lies in understanding what happens in the connections between neurons, in the way neurons pass information between each other. Possibly the most influential theory in neurobiology, the ionic hypothesis (proposed by Alan Hodgkin, Andrew Huxley, and Bernhard Katz), explains information flow through neurons as a change in electrical potential. The first step in understanding the neuronal basis for this behavior is to ask what the nerve cells are doing

Electrical impulses are the language of the nervous system. These tiny signals can be measured directly in single neurons of *Aplysia* by means of microelectrodes, hollow glass needles pulled to a fine tip (~10 μm) that are inserted into the neuron without killing it. The microelectrode contains a salt solution and

fine wire that connects to an amplifier. With another wire lead in the fluid surrounding the neuron, the miniscule difference in electrical potential between the inside of the cell and the outside can be accurately measured.

The cell's membrane, full of lipids, provides the insulating barrier for this 'membrane potential,' which amounts to about –70 millivolts (mV). The negative sign on the membrane potential is due to active pumps in the membrane that extrude positively charged sodium and calcium ions, and to the preponderance of negatively charged proteins inside the cell. Most importantly, this membrane potential changes when the nerve cell conducts an impulse.

Figure 10.2 shows that, when the gill is touched, specialized endings in the mechanosensory neurons generate an electrical signal that travels quickly down the length of the cell from the skin to the abdominal ganglion. To the microelectrode, the passing of this impulse looks like a spike as the membrane potential rapidly rises to +30 mV and then falls back down again. A microelectrode in one of the motor neurons shows their response to it several milliseconds later, and the muscle contraction follows a few milliseconds after that. Within each cell, these spikes (also called action potentials) are always roughly the same size. What varies is the number of them, which is proportional to the strength of the original stimulus to the gill.

Action potentials don't occur randomly, however; they have to be set off. At the sensory end, the membrane at the far end of the sensory neuron in the skin is sensitive to deformation so that when the skin is touched, an electrical potential (called an 'epsp' for 'excitatory postsynaptic potential') is generated locally at the site of the synapse where the sensory neuron contacts the motor neuron. If large enough, this epsp then triggers an action potential. In the motor neuron, the triggering event can be seen in the microelectrode recordings. Just prior to the rapid spike, there is a smaller and more prolonged potential change (Figure 10.3). Moreover, the size and duration of this slower potential varies with the strength of the original stimulus—the more the gill is stimulated, the more spikes are sent down the sensory neurons, and the larger the initial response in the motor neuron. This response, in turn, sets off the action potentials in the motor neurons.

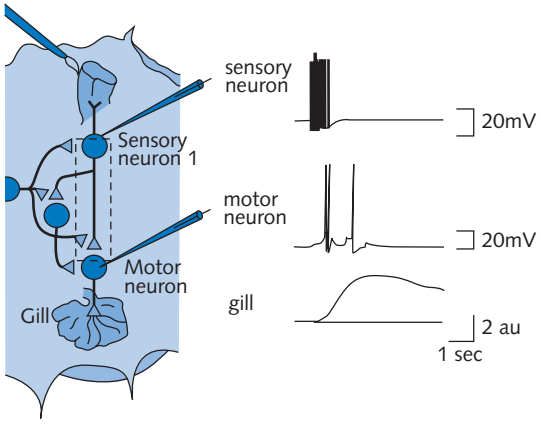

Figure 10.2 Action potentials in gill withdrawal.
Source: Kandel (1976).

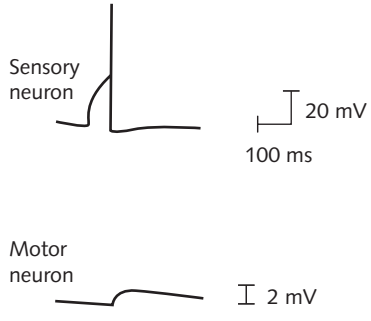

Figure 10.3 Sensory neuron action potential triggers epsp in motor neurons.
Source: Frost *et al.* (1985).

Action potentials move along neurons, but, generally speaking, they don't jump directly from one nerve cell to the next neuron in the chain. The synapse intervenes. Communication between neurons occurs at synapses and uses the same depolarization mechanism. However, this time it is not an electrical signal. Instead, there is a set of small molecules, known as neurotransmitters (for instance glutamate, serotonin, dopamine, and GABA) that are released from the end of the axon, diffuse over to the nearest part of another neuron (often called a dendrite), and bind to receptors. The effect is to open ion channels that initiate new electrical signals, which can then propagate a new action potential.

Key Papers

Albright, T.D., Jessell, T.M., Kandel, E.R., and Posner, M.I. (2000). Neural science: a century of progress and the mysteries that remain. *Neuron* **25** (Suppl.):S1–S55.

A historical overview of neuroscience.

Greenspan, R.J. (2007). *An Introduction to Nervous Systems*. New York: Cold Spring Harbor Laboratory Press.

As the title says, this is an introduction to nervous systems and we strongly recommend that you buy it.

Like Skinner, but for different reasons, Kandel wanted to do away with the brain. Could the learning processes so carefully characterized by behavioral psychologists in the first part of the 20th century—habituation, sensitization, classical and operant conditioning—be modeled with a neuronal circuit taken out of the whole organism? Natural sensory stimuli, the wave falling on the animal's skin, would be replaced by an artificial electrical or chemical stimulus. The response, gill withdrawal, would be replaced by a recording electrode to detect the action potential from the motor neuron that, in the normal course of things, would have been making its way to a muscle.

The idea was that neuronal pathways would be stimulated in an *in vitro* system, where nerve impulses would be under the control of the experimenter and the effects on other nerves could be monitored. Would synapses change systematically in response to different patterns of stimulation, and, if so, would the synaptic changes in any way parallel changes in the behavior of intact animals?

By substituting puffs of serotonin for tail shocks, it was possible to model a neural circuit in a dish of cultured neurons. While four or five spaced shocks give rise to a memory in the intact animal that lasts several days, five spaced puffs of serotonin have same effect in a culture dish full of neurons, making it possible to investigate the molecular basis of long-term memory. Serotonin acts via a group of enzymes (called kinases) that exert their effect on events in the cell by phosphorylating other proteins. So serotonin binds to receptors on the neuron's surface. These receptors in turn activate an enzyme (adenylyl cyclase) to produce a diffusible chemical signal (cyclic AMP), and cAMP binds to a protein kinase called PKA. PKA then phosphorylates yet another enzyme, mitogen-activated protein kinase (MAP kinase), and together the two of them act on a protein called CREB-1 (cAMP response element-binding protein), which is a regulator of gene activity in the nucleus. When CREB is phosphorylated, it enters the cell's nucleus and begins a process of altering which genes are active in that cell. In other words, it changes the proteins that the cell makes, which produces lasting changes to the cell's synapses (including growing new ones) and, as a result, a long-term form of sensitization. It is the formation of these new synapses that underlies long-term memory.

A typical sensory neuron has about 1,200 synapses; following sensitization, the number more than doubles.

Fruit flies remember things using the same cellular machinery discovered in sea snails

Soon after his circadian rhythm work, Benzer initiated a project to isolate new mutants that disrupted learning and memory in the fly, the genetic approach again at the forefront of attempts to understand the biology of behavior. Mutagenesis is a voyage into the unknown, promising to deliver something completely novel. But this promise needs to be tempered with the problem of understanding what is found at the end of the journey. The strength of the method is its power to find the completely unexpected as influences on behavior. But discovery comes at a price. When we have the answer, the mutation in the gene, we almost certainly won't know what that answer means. This is why we need to work with a model organism that can be used to do more than genetics. One of the surprises of the fly work was that genetic mechanisms appeared to operate in different species, so that what we learned from fruit fly genetics could be interpreted in the context of sea snail neurobiology.

Fruit flies can be trained to remember, to learn to tell the difference between smells or between things they see. If an odor (or image) is accompanied by an electric shock or a heat lamp, flies quickly learn to avoid it; the memory of this association will last for a day at most. What does it take to get them to remember it for longer? Regular conscientious practice (just like your piano teacher may have told you). In other words, as any student knows, cramming for an exam (i.e. all of the training at once), can get you through the next day, but it doesn't stick. Studying at regular intervals, on the other hand, produces a much longer-lasting memory. Fruit flies are no different. If multiple training trials are delivered all at once, memory still only lasts for a day. But if the training trials are interrupted with 15-minute rest intervals, then the flies will remember it for up to a week. This is a substantial portion of a fruit fly's lifespan – mid-life crisis has already set in by their second week of adulthood.

What does a fruit fly need to learn and remember in its brief, itinerant, polygamous life? Given the fact that the training regimens described above are never 100% effective, and that the flies must have the daylights shocked out of them to get above a 70% learning score, you might be tempted to say, 'Not much.' But it turns out that memory mechanisms are constantly used in the short term for tasks as simple as making a choice between two odors, or two food sources, or two females (if you are a male fly looking for a mate). The very act of making a discrimination requires the fly to remember one stimulus so that it can be compared with another.

Beyond the very short term, male flies also remember if they are soundly rejected by a female. This typically occurs if the male tries to court a female who has recently mated. (Courtship itself consists of a series of advances that males must make toward females, including tapping them, following them, extending one wing to 'sing' a courtship song, licking their

genitals, and mounting them, if the female permits.) If the female has mated and is unreceptive, she kicks the male repeatedly, buzzes her wings at him, and emits a pheromone that dampens his enthusiasm considerably. He will then not bother her again for hours afterward, but is not so crestfallen that he doesn't look elsewhere.

Mutagenizing flies, by chemically inducing single base-pair changes in their DNA (point mutations) is easy to do, but has two drawbacks. Large numbers of animals have to be screened for the behavior of interest and then the altered gene has to be cloned. It took roughly 5,000 lines of mutagenized flies, bred and behaviorally tested over several years, before Benzer, his colleagues, and various others were able to identify five mutants (Waddell and Quinn, 2001). Their criteria for learning-deficient mutants were: (i) during testing, the flies had to fail selectively to avoid the shock-associated odorant; and (ii) this failure could not be due to sensory or motor defects. In their first mutant screen, approximately 500 mutagenized X-chromosome lines were tested. About 20 lines met the first criterion; of these, only one also satisfied the second. The strain was made homozygous and was (for obvious reasons) named *dunce*.

dunce mutants forget more quickly than normal flies in the 30-minute period following conditioning; that is, they have defective short-term memory. This sieve-like memory is not confined merely to those two odors or to electric-shock-induced learning: it generalizes. *dunce* mutants rapidly forget odor cues associated with a food reward as well as with shock, they can't learn to discriminate visual patterns when heat is associated instead of an electric shock, and male *dunce* flies even fail to remember when a female has dumped them. In fact, by every conceivable criterion, *dunce* is a behavioral mutant due to an abnormality of its central nervous system.

By the turn of the century, ten *Drosophila* learning and memory mutants had been identified: *dunce*, *rutabaga*, *radish*, *amnesiac, latheo, linotte, nalyot, Volado* and *leonardo* (Waddell and Quinn, 2001). By 2007, the number had risen to more than 80 (Keene and Waddell, 2007). Who thought up those names? The answer is: whoever isolated the mutants. The choice is sometimes meaningful and sometimes less so. *dunce* and *amnesiac* are self-explanatory. *rutabaga*, *radish* and *turnip* were so named for being 'as dumb as a vegetable.' Latheo is the name of the 'river of forgetting' in Greek mythology that one must cross before entering Hades. *linotte* comes from the French expression 'tête de linotte,' which is comparable to 'bird brain.' Nalyot was one of Pavlov's dogs. Volado is Chilean slang for absent-minded, and *leonardo* is so named because it is a gene that is versatile (i.e. 'Renaissance') in its functions. Mutagenesis appears to have worked, giving reagents that potentially allow us to answer how genes influence behavior. What are these genes?

Molecular techniques make it possible to find out what these genes are, but, as we have stressed, the techniques are by no means easy. Gene cloning of point mutations in any organism, even when you have the complete genome sequence to hand, is an arduous affair, full of pitfalls. When *radish* was first cloned, it was said to be a mutation in a phospholipase gene. Then, in 2006, it was said to be in another gene, one with no name other than the identifier from the fly genome database: *CG15720* (Folkers *et al.*, 2006). Here's a quote from an article by one of the fly learning researchers that reviewed the evidence of genetic effects on learning and memory in the fruit fly *Drosophila*:

It also is worth noting that the nature of the molecular lesion associated with the *amnesiac* mutation remains unknown despite a report by Feany and Quinn to have found a point mutation in the *amnesiac* transcript [i.e. mRNA]. Moore *et al.* failed to detect any point mutation in *amnesiac* and claimed, rather, that the DNA sequence obtained from *amnesiac*+ flies (i.e., the normal control strain) of Feany and Quinn was in error, thereby producing an incorrect coding sequence of the *amnesiac*+ gene and an apparent point mutation in *amnesiac*. To date, this critical error in Feany and Quinn has not been clarified or corrected by the authors.

Margulies *et al.* (2005).

Oh well, *c'est la guerre*.

The first two genes identified from screens for learning and memory defects uncovered two components of a cAMP cascade: *dunce* encodes a cAMP phosphodiesterase and *rutabaga* encodes a type I Ca^{2+}/calmodulin-stimulated adenylyl cyclase. These are two components of the same cascade that serotonin acts on in the gill-withdrawal reflex. Furthermore, CREB turns out to be just as important in flies as it is sea snails: flies with deficiencies of CREB also have poor memory (Yin *et al.*, 1994). The cAMP pathway acts in mammals as well as invertebrates; mutations in CREB, for example, alter memory in mice (Bourtchuladze *et al.*, 1994) and there is a human mental retardation syndrome due to mutations in CREB-binding protein (CBP) gene (Petrij *et al.*, 1995).

There are a couple of odd things here. The first is that memory mechanisms are conserved between species. Is this really saying that my memories are laid down in the same way as a fly or a snail? We have to be precise about the level at which homology occurs: is it at the level of the gene, the transcript, the protein, the cell, the neural network, or the entire physiological process? At this stage, the best answer we can give appears to be that the homology occurs because neurons throughout the animal kingdom have common structural features. In a sense, what these experiments tell us about is how neurons work, rather than how brains function.

The secondly, slightly uncomfortable, observation is the need for the genetics experiments. Why bother with all that complicated mutagenesis, screening, and molecular cloning if the answer was there already from the *Aplysia* work? Of course, we'd have to test the prediction that the same genes were involved, but that is a much easier experiment than cloning induced mutants. One of the recurring lessons from gene cloning experiments is the need to have a physiological context, a mechanism, in which to interpret the finding (see Figure 10.4).

That said, one must take care not to ignore all of the other genes that mutagenesis in the fly has identified. The cAMP story is now only a small fragment of a much larger and still developing story. These other interesting genes include those for α-integrin, fasciclin II neural cell adhesion molecules and mRNA transport and translation control molecules encoded by the *pumilio* (another of Pavlov's dogs), *oskar* (named after the main character of Gunter Grass's *Tin Drum*, because when it is mutant in embryos, it produces a shortened body), and *eIF-5C* genes (Dubnau *et al.*, 2003). Undoubtedly, these, and the other 80 or so that have been identified but not fully characterized could tell us much more about the molecular process of memory. The problem of course remains as to how we set about understanding what those genes do. Perhaps another model organism would help here, one that permits a systematic analysis of genes, neurons, circuitry, and behavior.

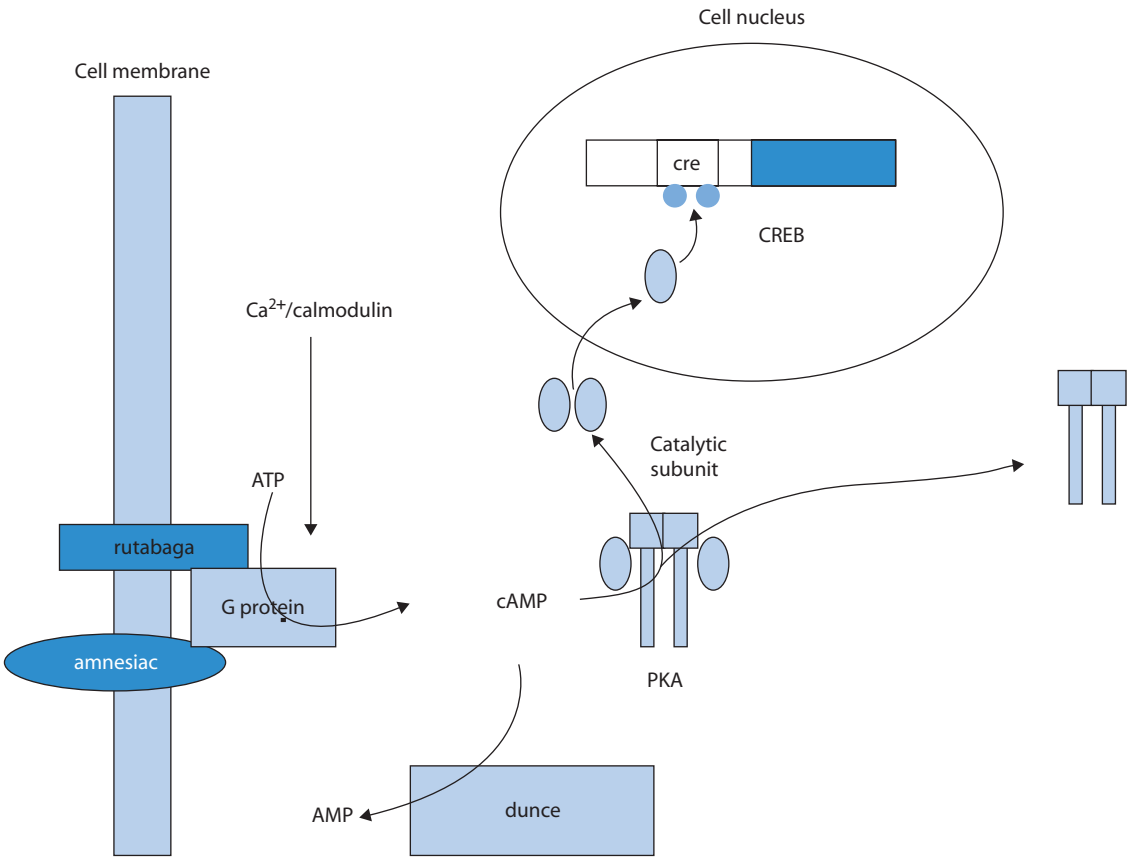

Figure 10.4 The cAMP signaling pathway involved in learning and memory in *Drosophila*. G-protein-coupled activation or Ca^{2+}/calmodulin stimulation leads to phosphorylation of CREB and activation of cAMP response element (cre)-linked genes in the cell nucleus. cAMP-dependent protein kinase A (PKA) mediates this signal. The *rutabaga* mutation affects cAMP signaling by disrupting a Ca^{2+}/calmodulin-dependent adenylate cyclase; the *amnesiac* mutation disrupts a protein that stimulates the cyclase; the *dunce* mutation affects the phosphodiesterase responsible for cAMP degradation.

The origins of genetic engineering as a tool for mammalian neurobiology

In 1999, newspaper reports announced the arrival of the Doogie mouse, the world's first genetically engineered super-intelligent animal (Tang *et al.*, 1999):

> **Scientists have boosted the intelligence of mice by adding a gene to the rodents' brains, and it should be possible to do the same in humans one day. Researchers at US universities found that adding copies of a specific gene to the mice significantly raised their ability to find their way through mazes, learn**

from objects and sounds and retain their new-found knowledge. They said the discovery could accelerate the development of medicines for human disorders such as memory loss in old age and Alzheimer's disease. But it could also sharpen an ethical debate about creating so-called 'designer children' with enhanced intelligence. The smarter mice have been nicknamed Doogie after a child prodigy character in the US television series 'Doogie Howser, MD'.

Agence France Presse, 2 September 1999.

Princeton University neurobiologist Joe Tsien, working with teams at Massachusetts Insitute of Technology and Washington University in St Louis, was quoted in the magazine as saying: '*They're learning things much better and remembering longer. They're smarter.*'

As it turned out, however, the mice may have learned faster, but they also forgot faster. The hype is typical of the impact of genetic engineering on mammalian neurobiology.

Synaptic plasticity: a cellular model of how memory works

Synapses are an obvious place to regulate the flow of information, a place where 'behavioral integration' could occur, in a phrase coined by Charles Scott Sherrington, one of the great forerunners of neuroscience. But without evidence that synapses actually regulate the flow of information in the nervous system, this idea remained speculative. Some neuroscientists, including Sherrington's contemporary, Karl Spencer Lashley, dismissed the idea entirely, saying '*…there is no direct evidence for any function of the anatomical synapse: there is no evidence that synapses vary in resistance, or that, if they do, the resistance is altered by the passage of the nerve impulse*' (Lashley, 1930). Others neuroscientists argued that understanding what happened at synapses was the key to understanding behavior. Donald Hebb provided one of the first, and still one of the most influential, accounts of how synapses were involved in learning and memory. He proposed that the information flow between neurons depended on to the amount of traffic (Hebb, 1949). Axons have many inputs, the dendrites, but only one output, the axon. With so many inputs, there are lots of opportunities for the neuron to fire off an action potential. But the neuron does not respond to every incoming message through its dendritic synapses. Hebb's suggestion was that the neuron will pay more attention to those synapses that are relatively more active. This is a cellular form of memory and the 'Hebb rule' has become a basic algorithm for learning. '*Let us assume then that the persistence or repetition of a reverberatory activity (or "trace") tends to induce lasting cellular changes that add to its stability.*' Hebb then states what has become widely known as 'Hebb's postulate': '*When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased*' (Hebb, 1949, p. 62).

In many ways, Hebb's idea (which has been summed up as 'cells that fire together wire together') was not novel; others had proposed that synaptic plasticity was a cellular correlate of behavioral modification. But Hebb wrote a whole book on the subject (a fairly unusual thing for a scientist to do, as rarely do their attention spans last longer than a three-page journal article). Writing a book also made it possible to deal systematically with the problems faced by his theory. So, for instance, a major

objection to his postulate was the existence of generalization. If we learn to discriminate a square from a circle or a cross, then we will automatically generalize that learning process, without further training: we recognize squares formed from dots or continuous lines, bigger or smaller than the original stimulus. Generalization without further training, which is true for rodents and for invertebrates, cannot be explained by strengthening of connections at a fixed and specific set of synapses.

Hebb could not ignore generalization, even though his neurophysiological postulate talks about plastic changes taking place at specific synapses resulting in more efficient conduction of impulses. His answer was to propose the existence of an '*irregular three-dimensional net*' that '*would be infinitely more complex than anything one could show with a diagram.*' These 'cell assemblies' consisted of strengthened associations between neurons across structurally modifiable synapses. Later, neuropsychologists would refer to similar assemblies as neural nets, but at the time there was no evidence for modifiable synapses, for the existence of synaptic plasticity, let alone one diffuse three-dimensional net.

Long-term potentiation: a cellular correlate of memory

The first evidence that synaptic plasticity existed in the brain came in the late 1960s and 1970s from both invertebrates, like *Aplysia*, and vertebrates, like frogs and rats. One such case that has become a primary focus of research is long-term potentiation (LTP). The first full description of LTP by Tim Bliss and Terje Lømo in 1973 reported that electrical stimulation to one neuronal pathway in the base of the rat brain (the perforant pathway in the hippocampus to be specific) caused a sustained increase in efficiency of synaptic transmission, consistent with a Hebbian form of synaptic plasticity. LTP is an artificial phenomenon: under normal circumstances, brains don't have electrical currents applied directly to their perforant pathways. However, the persistent changes in synaptic strength they elicited were immediately recognized as a potential model for learning and memory. How did it work?

A critical discovery was the involvement of specific neurotransmitter receptors in the induction of LTP. Graham Collingridge describes how this discovery was made. He had decided to explore the effect of glutamate, the major excitatory neurotransmitter in the brain, on LTP. Ionotropic glutamate receptors are channels in the cell membrane that open when glutamate binds to them, allowing ions of potassium, sodium, or calcium to travel through a central pore in the receptor, which makes a channel through the cell membrane. As it was known that the effects of glutamate are mediated by a number of different receptors, the question was which subtype (if any) might be involved.

> **There was no *a priori* reason to suspect one type of glutamate receptor over another with respect to a specific role in synaptic plasticity. I therefore decided to investigate the subtypes in a random order… The next agonist I tested was what I thought was N-methyl-DL-aspartate [NMDA], which had little effect in the CA1 region of the hippocampus. I felt unhappy with this result since my previous experiments in the substantia nigra had shown that NMDA was an extremely potent excitant of these neurons. Although this could obviously**

be due to a regional difference in sensitivity, I was sufficiently concerned to want to test *bona fide* NMDA, which at the time was not commercially available. On a visit to Bristol I raised my concern with Jeff Watkins who let me have samples, not only of NMDA but also two of his latest glutamate antagonists, (D, L)-2-amino-5-phosphonopentanoate (AP5) and DGG. On my return to Vancouver in the spring of 1981 I applied NMDA by ionophoresis to CA1 dendrites and observed dramatic effects.

Collingridge (2003).

NMDA and the drug that blocks its action, the antagonist AP5, were to be major players in the subsequent history of work on memory. While AP5 had no effect on synaptic transmission and no effect on LTP once it had been established, it blocked the induction of LTP in a reversible manner. Furthermore, using the same method, applying drugs that act on the receptors and drugs that block their action, Collingridge found that another, non-NDMA, glutamate receptor mediated synaptic transmission. This subtype is called the AMPA receptor. When it was discovered that magnesium ions block the NMDA receptor, and that this blockade is voltage dependent (so that depolarization will shift the magnesium out of the NMDA receptor), the idea emerged that the NMDA receptor was a coincidence detector. It works as follows (Figure 10.5): glutamate is released into the synaptic cleft (the gap between the two cells at a synapse) and arrives at AMPA receptors, which depolarize the membrane. Before depolarization, magnesium blocks the NMDA receptor from opening. Depolarization unblocks the magnesium and the NMDA receptor now opens in response to glutamate, allowing calcium to enter the cell, thereby making it much more responsive to subsequent stimuli, just the kind of change required for synaptic plasticity, the Hebbian basis of learning and memory.

The NMDA receptor story raises a set of questions about the molecular mechanisms of plasticity: how does calcium entry result in LTP? Is there a sensor at the mouth of the NMDAR

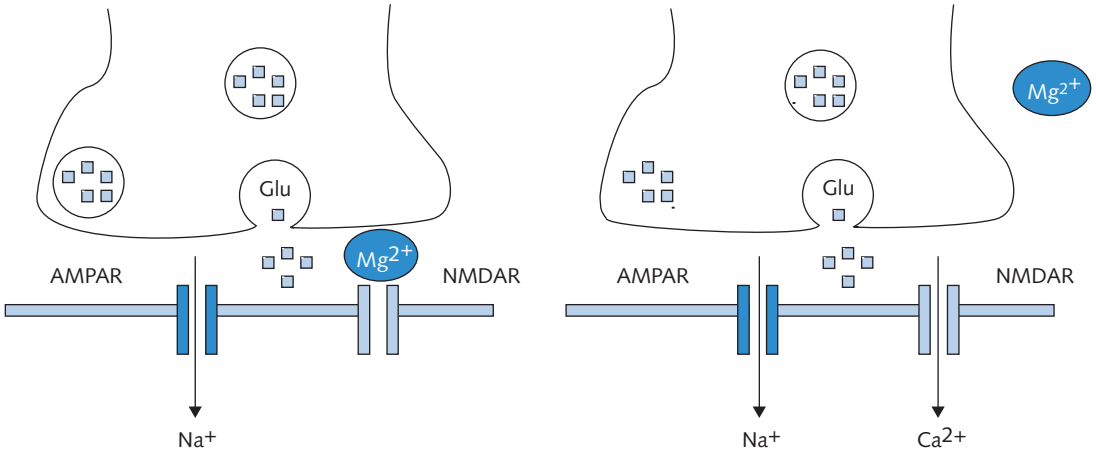

Figure 10.5 A simple view of potentiation at a synapse. Glutamate (Glu) is released into the synaptic cleft where depolarizes the membrane through its action on AMPA receptors (AMPAR). Before depolarization, magnesium ions (Mg^{2+}) block the NMDA receptors (NMDAR). After depolarization, the magnesium block is released and calcium (Ca^{2+}) can now enter the cell.

channel that triggers LTP or does Ca^{2+} have to diffuse further into the cell? What is that sensor? What intracellular signaling pathways does it activate and which are necessary for triggering LTP? These questions, and others like them, could be answered by gene targeting, but their importance to us is the light they cast on understanding the biology of behavior, or more specifically of memory.

Different forms of memory in the mammalian hippocampus

I (J.F.) have considerable sympathy for Karl Lashley. He died in 1958 with a reputation as one of the foremost psychologists of his day, despite a total lack of formal training in psychology (his first research was on very small creatures practically devoid of nervous systems, *Hydra* and *Paramecium*, at a time when it was acceptable to call them lower organisms). Lashley is famous for championing the view that all neural functions are distributed widely throughout the brain: '*…cerebral organization can be described only in terms of relative masses and spatial arrangements of gross parts, of equilibrium among the parts, of directness and steepness of gradients, and of the sensitization of final common paths to patterns of excitation*' (Lashley, 1930).

Lashley argued against phrenology and he also argued against synaptic plasticity: '*…there is no direct evidence for any function of the anatomical synapse: there is no evidence that synapses vary in resistance, or that, if they do, the resistance is altered by the passage of the nerve impulse.*'

Fifty years after Lashley's death every self-respecting psychology department practices the modern-day equivalent of phrenology, using functional magnetic resonance imaging to identify which parts of the brain are activated when we see, listen, talk, or think, or which parts of our brain are activated when we are sad, happy, angry, or disgusted; and every psychologist has learnt of the existence of long-term potentiation as a model of synaptic plasticity. What would Lashley have thought of the attempts to put these two beliefs together, to produce a theory that long-term potentiation in specific regions of the brain is responsible for our ability to remember?

Here is the core hypothesis, as the psychologist Richard Morris states it: '*Activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed*' (Morris *et al.*, 2003).

As Morris goes on to argue, the problem with this hypothesis is that it lacks specificity. Synaptic plasticity has many forms, but, perhaps more importantly, memory is not a unitary phenomenon. For instance, there is a distinction between the unconscious memory for perceptual and motor skills (called implicit or procedural memory) and conscious memory of things, places, and past events (termed explicit or declarative memory). This distinction has good anatomical justification, as became evident with the publication in 1957 of a case report of a man, referred to as H.M., with permanent damage to a region of the brain called the hippocampus.

There's a hotel, a little further up the road from where I'm staying as I write this, called The Hippocampus. The hotel sign shows a badly

weathered painting of a sea horse. I've never seen any resemblance between the convoluted bilateral brain structure, either in rodents or humans, and a sea horse, but apparently somebody did. And now probably more psychologists study the hippocampus than any other brain region, so many in fact that there is a journal devoted to presenting their findings, a journal called, appropriately, *Hippocampus*. Consequently, the hippocampus is one brain region whose functions are relatively well understood.

H.M. showed that without a hippocampus you could still talk and think normally and could still learn new motor tasks (intact implicit memory), but you could not learn new facts. Furthermore, while H.M. could remember distant events—his childhood for example—he could not remember events recently before (or since) the onset of amnesia. All these observations suggested that the hippocampus was involved in processing declarative memory, or at least two components of declarative memory: the ability to memorize facts (semantic memory) and the ability to memorize personal experiences (episodic memory), that is to say the memories of what happened to me and when (the distinction between semantic and episodic memory is the difference between knowing that the hippocampus is part of the brain, and knowing when you learnt that bit of information).

Claims that the hippocampus was essential for declarative memory were surprising, because at that time there was no evidence that animals with lesions to the hippocampus suffered amnesia, or that they could not remember new tasks. Psychologists then working in the phrenological tradition investigated brain function by destroying parts of the brain and searching for behavioral abnormalities. This was work Lashley knew well, and it showed that animals with damage to the hippocampus had difficulty suppressing responses that were no longer appropriate. For instance, among the lever-pressing tasks that psychologists liked to teach rats was one that required the animal to delay the rate of lever pressing to obtain a reward (for example leave an interval of at least 5 seconds between lever presses to get the food). Rats with damage to the hippocampus always found this task difficult, and the greater the damage to the hippocampus, the worse the deficit. Thus, until about 1970, the prevailing hypothesis of hippocampal function from animal work was that the hippocampus mediated behavioral inhibition.

John O'Keefe and John Dostrovsky fundamentally changed that view. In 1971, they found that the pyramidal cells of the hippocampus, the same cells later to be stimulated electrically in the discovery of LTP, encode where a rat is: the firing pattern of pyramidal neurons is an internal spatial map (O'Keefe and Dostrovsky, 1971). Within minutes of placing a rat in a new environment, it develops a stable internal representation of the space, such that a pyramidal cell's firing pattern depends on the animal's location: for example, after the rat is placed in a box, specific hippocampal cells will fire when the rat is in the northwest corner, others when it is in the southeast corner and so on. Pyramidal place fields remain stable for weeks if the environment is constant, consistent with the idea that the cells contribute to a particular spatial memory.

If one role of the hippocampus is to make and remember maps, and if the only contender for the cellular basis of memory, LTP, is found in the hippocampus, a critical experiment is to determine whether the two are causally related. Richard Morris developed a spatial memory task, now forever known as the Morris water maze, which could be used to investigate the relationship between memory and LTP. Rats were used for this work and rats are

good swimmers, although they are not fond of water. As I can personally testify from trying to wash a pet rat in a sink, rats swim fast and make desperate attempts to climb out when they are put in water.

The Morris water maze isn't a maze at all, at least not the sort of maze I think of, one with large walls, usually made of a dense dark green shrub, forming passages that lead off into blind alleys or back to where you started from, the whole thing designed to confuse and disorientate you so that you lose all idea of where you are and, if the maze is good, stop you from leaving it. Richard Morris' water maze is a large circular pool, about five feet in diameter, containing what looks like milk (the water is made opaque so the animal can't see what's hidden under the surface). The sides are vertical and tall enough to stop the animal jumping or climbing out. The only refuge is one small region of the pool where the water is very shallow, and there is a small platform that the rat can stand on. Using water to hide the platform means the rat can't use smell to identify it. When a rat is put in the pool, it swims around until by chance (it cannot see the platform) it reaches relative safety. Replace the rat in the pool and the animal finds the platform a little quicker as it now has some idea of the hidden location. Keep training the rat in this way and it will eventually learn where the platform is, swimming quickly towards it. By starting the rat randomly at different points in the pool (south, east, north, or west) and providing fixed visual cues (shapes such as stars and squares stuck onto the poolside) as spatial reference points, it's possible to ensure that the only way the rat can solve the maze is by remembering the platform's spatial position, so that the animal might be saying to itself that the platform is to the left of the star and to the right of the square, just as we would use a map to triangulate on a position.

The psychologists' way of asking the rat if it knows the platform's location is to remove the platform, which is of course invisible, and then put the rat into the pool. The rat with intact spatial memory will swim to where it thinks the platform is located and persist in swimming over the missing platform (you can imagine what it must be thinking). A rat that doesn't remember, or hasn't learnt the task, may swim over the missing platform, but won't waste time swimming in pointless circles around one region.

Richard Morris used AP5, the same drug that Graham Collingridge had used to block NMDA receptors, to turn off LTP in the hippocampus. Infusing AP5 into the brain impaired spatial learning in the water maze. Using two versions of the maze, one with a hidden platform below water and one with a visible platform, Morris showed that drugged animals failed to learn the location of the hidden platform only, indicating that NMDA receptor inactivation specifically inhibited spatial learning. This selectivity of effect on learning the task implies a direct action on remembrance.

Morris's paper was one important piece of evidence for a relationship between LTP and spatial memory. However, as Morris points out, these experiments do not prove the assumption that AP5 causes its effects on learning and memory by blocking hippocampal synaptic plasticity. '*The logical difficulty is as follows: blockade of LTP by AP5 is a dependent consequence of the drug treatment. The impairment of learning is also a dependent consequence. However it is fallacious to presume that that one dependent consequence is necessarily the cause of the other*' (Morris *et al.*, 2003). In other words, more direct tests are needed to establish the relationship between NMDA receptors, LTP, and learning and memory. This is what the genetic experiments, described next, set out to do.

Key Papers

O'Keefe, J. and Dostrovsky, J. (1971). The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Res* **34**:171–175.

O'Keefe, J. and Nadel, L. (1978). *The Hippocampus as a Cognitive Map*. Oxford: Clarendon Press.

Morris, R.G., Anderson, E., Lynch, G.S., and Baudry, M. (1986). Selective impairment of learning and blockade of long-term potentiation by an *N*-methyl-D-aspartate receptor antagonist, AP5. *Nature* **319**:774–776.

Knocking out memory in the mouse

It was not immediately obvious, at least not to me, why the first genes to be targeted in neurobiology were kinases. Why not go for the receptors, the AMPA and NMDA receptors that we know to be important? I asked Seth Grant, one of the first people to do this work, to explain. One reason was practical: knockouts for some kinases had already been made, by cancer biologists, and the genes for others had been identified. As Grant put it, there were good cancer reagents. By contrast, knocking out the receptors meant cloning the DNA to make the targeting construct, and then targeting the ES cells and so on.

One reason why NMDA receptors weren't the first genes to be targeted was because they weren't cloned until 1991 and the full complexity of their structure was not appreciated at the time. As it turned out, deleting all NMDA receptors was lethal because they are used in the midbrain to control breathing. As cancer biologists had shown that knocking out kinase genes did not kill mice, and that the mutants were useful reagents for understanding cancer, they were a promising starting point. The question was whether cancer reagents would be useful for neurobiology.

But why would anyone expect kinases to be involved in long-term potentiation? Kinases are proteins that add phosphates to proteins; phosphatases remove phosphates from proteins. Nobel laureate Paul Greengard had shown how critical phosphorylation reactions were for neurotransmitter function. He had shown that slow signaling pathways modulate fast synaptic transmission in two major ways: '*a) by regulating the state of phosphorylation of synapsins and other key proteins present in the pre-synaptic terminal, thus modulating the efficacy of neurotransmitter release (the amount of neurotransmitter released from the nerve terminal in response to an action potential), and b) by regulating the state of phosphorylation of neurotransmitter receptors present in the post-synaptic cell, thus modulating the responsivity of these receptors to the released neurotransmitter (responsivity referring to the magnitude of the electrophysiological response to a molecule of neurotransmitter)*' (Greengard, 2001).

The reversibility of the kinase/phosphatase reaction had been a contender as a key component of memory for some time, because of the possibility that it could act in a switch-like manner (Lisman, 1985). One kinase had already been proposed to take on this role: calcium/calmodulin protein kinase II (CamKII). CamKII is present in great abundance in neurons (up to 2% of total protein), which is unusual for a regulatory protein, typically present in very small amounts. As its name suggests, the kinase activity of CamKII depends on the levels of calcium in a cell bound to a protein

called calmodulin. When calcium/calmodulin binds to CamKII it turns on the kinase, but this dependence on its activator can be bypassed: active CamKII can add phosphate to itself, and when it does so it remains active in the absence of calcium/calmodulin. It can only be turned off by the action of a phosphatase. This is the switch-like property that caught people's attention and suggested that CamKII might be a memory molecule. And NMDA receptors let calcium into the cell. Calcium had to act somewhere to change the cell's responsivity and CamKII was a good starting point.

Seth Grant, believing that kinases had to be important in transforming the signal from the NMDA receptor into LTP, set out to look at mice with deleted kinases. Working with Eric Kandel and Tom O'Dell, he began to examine the knockouts and found that one mutant, for *fyn* kinase, did indeed show a learning abnormality. At the same time, Alcino Silva, working with Susumu Tonegawa at the Massachusetts Institute of Technology, used gene targeting to show that CamKII was required for the induction of LTP. Both Silva and Grant also showed that the knockout animals were deficient in spatial memory, using the Morris water maze.

Key Papers

Grant, S.G.N., O'Dell, T.J., Karl, K.A., Stein, P.L., Soriano, P., and Kandel, E.R. (1992). Impaired long-term potentiation, spatial learning and hippocampal development in fyn mutant mice. *Science* **258**:1903–1910.

Silva, A.J., Paylor, R., Wehner, J.M., and Tonegawa, S. (1992). Impaired spatial learning in a calcium–calmodulin kinase II mutant mice. *Science* **257**:206–211.

Two key papers that introduced molecular biology to psychologists.

Seth Grant has stressed to me that his paper reported results not on one knockout but on four (a reasonable thing to stress given the amount of work that involved). The other thing he stressed is less obvious: the knockouts did learn to find the hidden platform in the water maze. At first sight, this is at odds with the summary I've given above, but there are a couple of things to explain here. Firstly, the 129 strain of mouse tends to develop tumors (a propensity for which it was developed in the first place) and it may have structural brain abnormalities (which might confound behavioral testing), so typically researchers cross the mutant animal with a more congenial strain (C57BL/6). The offspring of the cross are no longer on a pure strain background, and there will be lots of genetic variants now segregating that affect all aspects of the animals' physiology and behavior. Some may, and in this case did, influence the effect of the mutation, reducing its impact and in fact restoring the animals' spatial learning ability. The importance and relevance of such background genetic effects were to provoke considerable discussion in later years. Seth Grant's report is one of the first to document them, as he found that the behavioral abnormality was only observed when the mutations were placed on the pure 129 strain. The second point is that the worst behavioral consequence of the mutation was seen when the knockout had to learn a new hidden position for the platform in the water maze (sometimes called a reversal learning task): for example, train the animal to find a platform hidden in the south-east corner of the pool and then try to train it to find a platform hidden in the north-west corner. This suggests the mutants are sensitive to a change in contingency. The importance of this observation, and the use of mutants to sort out what was going on, will become clearer in due course.

Mutant mysteries

I've no idea why he did this experiment. It's always seemed completely counter-intuitive why anyone should think of it in the first place. None the less, the experimental psychologist David Bannerman carried out what is known as a two-pool experiment. After training a rat to find a fixed-position platform or a varying-position platform in one room, he put the animal into another room and trained it again. Pre-trained animals were not affected by AP5 (the drug that abolishes NMDA receptor function). Why does pre-training make a difference?

Gene-targeting experiments added to the mystery. After the glutamate receptors had been cloned, the knockouts could be created. NMDA receptor knockouts initially proved useless because the homozygous mutant is lethal—not surprising given the receptor's critical function in the midbrain. But by the mid-1990s, a way around this impediment was found. Using a recombinase (an enzyme that cuts DNA), it is possibly to delete a gene in the adult animal, just in the hippocampus. Joe Tsien in Tonegawa's laboratory did this for the NMDA receptor, knocking out one component of the receptor in one region of the hippocampus and replicated the AP5 results: no LTP and no spatial learning in the water maze (Tsien *et al.*, 1996). However, another mutant, restricted to a different hippocampal region, performed normally in the water maze (Nakazawa *et al.*, 2002), but were severely impaired in the reversal learning version of the water maze (where the animal has to relearn different platform locations in the same apparatus).

An even bigger challenge to the new orthodox interpretation of the molecular biology of memory arose with analysis of a different glutamate receptor mutant. A knockout of the AMPA receptor yielded one of those rare, completely unexpected results that turn out to be remarkably informative. AMPA receptors have four subunits (called GluR1 to GluR4, but also called GluR-A to GluR-D, so expect to be confused if you start reading the original literature on this subject). The adult hippocampus contains receptors made up of GluR2 bound to either GluR1 or GluR3. Knocking out GluR1 is not lethal, but it does abolish LTP in the hippocampus. The mutation should also, according to the hypothesis that LTP is a cellular correlate of memory, prevent an animal from learning to find the hidden platform in the water maze. It doesn't. Mutants learn just as well as their brothers and sisters with intact GluR1 genes (Zamanillo *et al.*, 1999).

A solution emerged with a re-analysis of the GlurR1 mutant on a new task (Reisel *et al.*, 2002). Place the animal at the bottom of an apparatus shaped like a capital T and allow it to run forward to the junction. As we discussed in Chapter 7, the rodent has to make a choice, left or right arm. Once it has chosen, start it over from the beginning again and score which arm it goes into. The average mouse (or rat) will choose a different arm, presumably because it knows there is no point in visiting the same arm twice (no food to find there). For the short time between the first and second task, the animal has to remember which arm it chose first. This type of memory, working memory (or to be more correct spatial working memory because it turns out there are different sorts of working memory) is severely impaired in the GluR1 knockout. This result says that you don't need working memory to learn the usual water maze task; not surprisingly, the knockouts deficient in working memory are impaired on the reversal

learning tasks (as these are taxing their ability to remember where they've been a few minutes ago).

Finding and interpreting such specificity of genetic action is a remarkable achievement. It illustrates how behavioral and genetic analyses can work together to find out how genes influence behavior. Only with carefully designed psychological paradigms can we tease apart the effects of individual genes on our behaviors of interest. But I think it is worth returning to a remark by Paul Greengard to remind ourselves how difficult this cooperation between different scientific fields usually is.

> **At the time we started this work, neuroscience was not a clearly defined field. There were two types of people studying the brain. There were biophysicists, working in physiology departments, who believed that everything significant about the brain could be explained in terms of electrical signaling. And there were biochemists working in biochemistry departments who would happily throw a brain into a homogenizer, with as much abandon as they would a liver, and look for enzymes or lipids. But these biochemists were rarely interested in brain function. And so these two groups rarely spoke to each other, which is just as well because when they did they didn't have nice things to say.**

Nowadays there are molecular biologists, who redesign the genomes of mice and speak a newly minted language, which only they understand, and behavioral neuroscientists who test mice in paddling pools and use the vocabulary of learning theory (which personally I think is equally impenetrable). The two groups do speak to each other, but it has to be said, not much.

Summary

1. Aplysia, a sea snail, is a model organism used to dissect the cellular and molecular basis of memory. The animal can be used to study forms of short- and long-term memory.

2. Memory in *Aplysia* is in part mediated by a cAMP signaling pathway in which serotonin receptors activate an enzyme (adenylyl cyclase) to produce a diffusible chemical signal (cyclic AMP). One consequence is the activation of CREB-1 (cAMP response element-binding protein), a regulator of gene activity in the nucleus.

3. Fruit flies have long- and short-term memory, also mediated by a cAMP signaling pathway. Key players in the pathway have been found by using mutagenesis.

4. The cAMP signaling pathway is also involved in mammalian learning and memory.

5. Synaptic plasticity is a form of cellular memory in which neurons learn to pay more attention to those synapses that are relatively more active.

6. Long-term potentiation (LTP), a form of synaptic plasticity, is believed to be a cellular correlate of spatial memory. LTP is an artificial phenomenon, evoked by electrical stimulation to one neuronal pathway in the base of the mammalian hippocampus. LTP is the sustained increase in efficiency of synaptic transmission, consequent upon stimulation.

7. Glutamate, an excitatory neurotransmitter, activates AMPA and NMDA receptors in the hippocampus. The NMDA receptor acts as a coincidence detector and is involved in LTP.

8. Evidence from drug effects and mutants indicates that NMDA receptor inactivation inhibits spatial learning.

9. Mutations in the AMPA receptor disrupt LTP but not spatial learning in the Morris water maze. However, the genetic lesion severely impairs spatial working memory. This is a rare example of specificity of genetic action on behavior.

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